Full Paper

The Cannabinoid Receptor Agonist WIN 55212-2 Inhibits Neurogenic Inflammations in Airway Tissues

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Abstract. We examined the effects of a cannabinoid receptor agonist, (*R*)-(+)-[2,3-dihydro-5-methyl-3-[(4-merpholino)methyl]pyrrolo-[1,2,3-*de*]-1,4-benzoxazin-6-yl](1-naphthyl)methanone (WIN 55212-2), on various respiratory reactions induced by the activation of capsaicin-sensitive afferent sensory nerves (C-fibers). WIN 55212-2 significantly inhibited capsaicin-induced guinea pig bronchoconstriction, but not the neurokinin A-induced reaction. Intravenous injection of WIN 55212-2 also blocked cigarette smoke-induced rat tracheal plasma extravasation. However, substance P-induced rat tracheal plasma extravasation was not affected by the administration of WIN 55212-2. A cannabinoid CB₂ receptor antagonist, {*N*-[(1*S*)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide} (SR 144528) reduced the inhibitory effects of WIN 55212-2, but not a cannabinoid CB₁ antagonist, [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamidehydrochloride] (SR 141716A). A Maxi-K⁺ channel opener, 1-(2'-hydroxy-5'-trifluoromethylphenyl)-5-trifluoromethyl-2(3*H*)benzimidazolone (NS 1619), specifically inhibited capsaicin-induced guinea pig bronchoconstriction and cigarette smoke-induced rat tracheal plasma extravasation. These findings suggest that WIN 55212-2 inhibits the activation of C-fibers via cannabinoid CB₂ receptors and Maxi-K⁺ channels and reduces airway neurogenic inflammatory reactions in vivo.

Keywords: cannabinoid, C-fiber, bronchoconstrictor, plasma extravasation, Maxi-K⁺ channel

Introduction

Several previous studies showed that neurogenic inflammation in the airway must have an important role in the pathogenesis of asthma (1, 2). By using tachykinin antagonists, Murai et al. (3, 4) directly demonstrated that airway inflammation is generated by tachykinin released from capsaicin-sensitive afferent sensory nerves (C-fibers), which are stimulated by various types of irritants like cigarette smoke (5), cold air (6), and hypertonic saline (7). These irritants activate C-fibers by opening non-selective cation channels with a high Ca²⁺ permeability (8). Morimoto et al. (9) showed that ω -conotoxin

GVIA blocks the activation of C-fibers and the release of substance P from their endings, and they suggested that the opening of N-type voltage-dependent Ca²⁺ channels might activate C-fibers in guinea pig airway tissues.

Cannabinoids are a direct class of psychoactive compounds that produce a wide array of effects including hypothermia, depressed motor activity, hypotension, inhibition of intestinal motility, and antinociception (10). The biological effects of cannabinoids are mediated by specific cell surface receptors, CB₁ and CB₂, in the central nervous system and in peripheral tissues (11–13). Over the last 10 years much evidence has accumulated favoring the concept that cannabinoids are endogenous modulators of neuronal activity in several tissues (14, 15). Cannabinoid receptor agonists have been shown to inhibit the function of Ca²⁺ channels (16,

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17). In our previous study, we discovered that a synthesized cannabinoid receptor agonist, WIN 55212-2, inhibited the activation of C-fibers and the release of these neuropeptides from their endings via the opening of Maxi- K^+ channels by the activation of CB_2 receptors with in vitro experiments (18); and it will be a valuable tool for the therapy for airway inflammatory diseases such as asthma.

In this report, we examined the effects of WIN 55212-2 on the activation of C-fibers in guinea pig and rat airway tissues in vivo and suggested that WIN 55212-2 reduced airway inflammatory reactions through the same mechanism as in vitro.

Material and Methods

Materials

Substance P and neurokinin A were purchased from Peptide Institute, Inc. (Osaka). Capsaicin was from Nakalai Tesque Chemical Company (Kyoto). 1-(2'-Hydroxy-5'-trifluoromethylphenyl)-5-trifluoromethyl-2(3H)benzimidazolone (NS 1619) was purchased from Funakoshi Chemical Co. (Tokyo). (R)-(+)-[2,3-Dihydro-5-methyl-3-[(4-morpholino)methyl]pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl](1-naphthyl)methanone (WIN 55212-2) was synthesized at Fujisawa Pharmaceutical Co., Ltd. (Osaka). [N-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide-hydrochloride] (SR 141716A) and {N-[(1S)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide} (SR 144528) were kindly supplied by Sanofi-Synthelabo Recherche (Montpellier, France). All drugs prepared in dimethylsulfoxide were injected intravenously (0.1 mL/kg). The solvent alone or dimethylsulfoxide had no effect on the responses in the in vivo studies.

Experimental animals

All experimental protocols were approved by the Animal Care and Use Committee of Dokkyo University School of Medicine and Fujisawa Pharmaceutical Co., Ltd. and were conducted in accordance with the guidelines of the National Institutes of Health and The Japanese Pharmacological Society.

Twelve-week-old male Hartley guinea pigs (280–340 g) and 6-week-old male Wistar rats (190–230 g) were purchased from SLC (Hamamatsu) at least 1 week before the experiments. Animals were housed in a temperature- and humidity-controlled environment under a 12:12 h light/dark cycle with light on at 7:00 A.M. Animals were allowed free access to food and water ad libitum.

Bronchoconstriction in guinea pigs in vivo

Male Hartley guinea pigs were anesthetized by intraperitoneal injection of α -chloralose (100 mg/kg) and urethane (500 mg/kg). The jugular vein and trachea were cannulated and the animals were ventilated artificially (5 mL of air, 60 strokes/min). The pressure in the respirator system, that is, the insufflation pressure, was measured constantly using a transducer (TP 200T; Nihon Kohden, Tokyo). Each stimulant was injected intravenously every 15 min through the juglar vein cannula to induce bronchoconstriction. Stimulants were administered repeatedly (approximately 6 times) until reproducible constriction was obtained. The bronchoconstriction after each stimulation returned to the basal level within 15 min after the stimulation. WIN 55212-2 and NS 1619 were dissolved in dimethylsulfoxide and administered intravenously (0.1 mL/kg) 2 min before the further challenge was repeated. The control response was taken at 15 min before the administration of WIN 55212-2 or NS 1619 in each experiment. After the administration of WIN 55212-2 or NS 1619 at 0 min, the bronchoconstriction induced by the injections of neurokinin A or capsaicin were measured at 2, 17, 32, 47, 62, 77, 92, and 107 min. We verified that constant bronchoconstricting responses were induced during 8 times repeated stimulations at each interval of 15 min (data not shown). Cannabinoid antagonists were also dissolved in dimethylsulfoxide and administered intravenously (0.1 mL/kg) 10 min before WIN 55212-2 injection. Administration of all chemicals and vehicle used in this study had no effect on the basal bronchoconstriction. The resulting constriction was compared with the control constriction. The following stimulants were used at the doses indicated: neurokinin A (1 nmol/kg) and capsaicin (10 nmol/kg).

Tracheal plasma extravasation in rats in vivo

Male Wistar rats were injected with Evans Blue dye (20 mg/kg, dissolved in saline) into a tail vein. Rats were immediately either injected with a chemical mediator or exposed to cigarette smoke. Substance P (32 nmol/kg) was dissolved in saline and injected into a tail vein. The cigarette exposure regime involved intense passive smoke exposure and is a modification of a previous procedure (19). Smoke from one third of a cigarette was delivered into cabinets in which the rats were temporarily housed. After 10 min, the animals were killed by exsanguination and the lungs were perfused with 50 mL of saline. The trachea and stem bronchi were dissected out, weighed, and then dissolved in 0.25 mL of 1 N KOH at 37°C for 6 h. After the extraction with 2.25 mL of acetone-phosphate solution (0.6 N H_3PO_4 : acetone = 5 : 13), the Evans Blue dye content of

tissues was quantified colorimetrically at 620 nm. WIN 55212-2 and NS 1619 were dissolved in dimethylsulfoxide (0.1 mL/kg) and injected into the rat tail vein 2 min before Evans blue dye injection. Injection of the vehicle used to dissolve WIN 55212-2 and NS 1619 had no effect. The increase in the amount of leaked Evans Blue dye was calculated by subtracting the value for Evans Blue solution without agonist ($15.2 \pm 0.8 \mu\text{g/g}$ tissue, $n = 5$).

Statistical analyses

Results are each given as the mean $\pm$ S.E.M. of five experiments. Statistical analyses were performed by either analysis of variance followed by Dunnett's multi-comparison test or by means of the unpaired Student's *t*-test (others). $P < 0.05$ was considered as indicative of significance.

Results

Effects of WIN 55212-2 and NS 1619 on bronchoconstriction in guinea pigs

We examined the effects of the cannabinoid receptor agonist WIN 55212-2 on capsaicin-induced bronchoconstriction in guinea pig airway in vivo. Intravenous administration of WIN 55212-2 (1.0–10.0 mg/kg) dose-dependently inhibited capsaicin-induced constriction (Fig. 1). However, neurokinin A-induced guinea pig bronchoconstriction was unaffected by WIN 55212-2 at a dose of 10.0 mg/kg (data not shown). To identify the cannabinoid receptor subtype that was involved in these

Fig. 1. Effects of a cannabinoid receptor agonist, 1.0 mg/kg (triangles), 3.2 mg/kg (squares), or 10.0 mg/kg (circles) WIN 55212-2, on capsaicin-induced guinea pig bronchoconstriction. Significantly different from the control constriction, * $P < 0.05$, ** $P < 0.01$. Values are given as % inhibition of the control constriction and are the mean $\pm$ S.E.M. of five experiments. The respective control bronchoconstriction was $22.3 \pm 3.2 \text{ cmH}_2\text{O}$ for triangles, $21.6 \pm 2.1 \text{ cmH}_2\text{O}$ for squares, and $23.2 \pm 1.8 \text{ cmH}_2\text{O}$ for circles.

effects of WIN 55212-2, we examined the influences of selective cannabinoid CB_1 (SR 141617A)- and CB_2 (SR 144528)-receptor antagonists. SR 144528 (0.1 mg/kg) reduced the inhibitory effect of 10.0 mg/kg WIN 55212-2 on capsaicin-induced guinea pig bronchoconstriction, but not 0.1 mg/kg SR 141617A (Fig. 2). A Maxi- K^+ channel opener, NS 1619, also inhibited capsaicin-induced bronchoconstriction at a dose of 1.0 mg/kg, but not neurokinin A-induced bronchoconstriction (Fig. 3). Neither WIN 55212-2 nor NS 1619

Fig. 2. Effect of 10.0 mg/kg WIN 55212-2 on capsaicin-induced guinea pig bronchoconstriction with cannabinoid receptor antagonists, 0.1 mg/kg SR 141617A (squares), 0.1 mg/kg SR 144528 (closed circles), or vehicle (open circles). Significantly different from vehicle, * $P < 0.05$, ** $P < 0.01$. Values are given as % inhibition of the control constriction and are the mean $\pm$ S.E.M. of five experiments. The respective control bronchoconstriction was $23.6 \pm 2.6 \text{ cmH}_2\text{O}$ for squares, $24.2 \pm 1.8 \text{ cmH}_2\text{O}$ for closed circles, and $23.6 \pm 1.6 \text{ cmH}_2\text{O}$ for open circles.

Fig. 3. Effects of 1.0 mg/kg NS 1619 on capsaicin-induced (open circles) and neurokinin A-induced (closed circles) guinea pig bronchoconstriction. Significantly different from the control constriction, * $P < 0.05$, ** $P < 0.01$. Values are given as % inhibition of the control constriction and are the mean $\pm$ S.E.M. of five experiments. The respective control bronchoconstriction was $22.3 \pm 3.2 \text{ cmH}_2\text{O}$ for open circles and $22.8 \pm 1.4 \text{ cmH}_2\text{O}$ for closed circles.

altered baseline airway resistance (% inhibition of baseline: 10.0 mg/kg WIN 55212-2, 1.3 ± 0.2 ; 1.0 mg/kg NS 1619, 1.2 ± 0.4 , $n = 5$).

Effects of WIN 55212-2 and NS 1619 on plasma extravasation induced by cigarette smoke in rat trachea

The effects of WIN 55212-2 on rat tracheal plasma extravasation induced by cigarette smoke were examined. Intravenous injection of WIN 55212-2 (0.01–0.1 mg/kg) dose-dependently inhibited cigarette smoke-induced rat tracheal plasma extravasation (Fig. 4). However, substance P-induced tracheal plasma extravasation was not affected by WIN 55212-2 at a dose of 0.1 mg/kg (Table 1). SR 144528 (0.1 mg/kg) reduced the inhibitory effect of 0.1 mg/kg WIN 55212-2 on cigarette smoke-induced guinea pig rat tracheal plasma extravasation, but not 0.1 mg/kg SR 141617A (Table 2). NS 1619 (1 mg/kg) injected intravenously, reduced significantly cigarette smoke-induced rat tracheal plasma extravasation, but not substance P-induced reaction (Table 3). Neither WIN 55212-2 (0.1 mg/kg) nor NS 1619

(1 mg/kg) itself altered basal vascular permeability in rat trachea (data not shown).

Fig. 4. Effects of WIN 55212-2 on cigarette smoke-induced rat tracheal plasma extravasation. Significantly different from the control extravasation, * $P < 0.05$, ** $P < 0.01$. Values are given as the mean $\pm$ S.E.M. of five experiments.

Table 1. Effect of WIN 55212-2 on cigarette smoke-induced and substance P-induced rat tracheal plasma extravasation

		Evans blue dye contents ($\mu\text{g/g}$ tissue)	
		Cigarette smoke	Substance P
Control		40.9 ± 2.2	41.2 ± 2.1
WIN 55212-2	0.1 mg/kg	$16.4 \pm 3.2^{**}$	37.1 ± 2.8

**Significantly different from vehicle, $P < 0.01$. Values are given as the mean $\pm$ S.E.M. of five experiments.

Table 2. Effect of WIN 55212-2 on cigarette smoke-induced rat tracheal plasma extravasation with cannabinoid receptor antagonists

		Evans blue dye contents ($\mu\text{g/g}$ tissue)		
		Vehicle	0.1 mg/kg SR 141716A	0.1 mg/kg SR 144528
Control		40.9 ± 2.2	42.6 ± 3.1	39.6 ± 1.8
WIN 55212-2	0.1 mg/kg	$16.4 \pm 3.2^{**}$	$20.3 \pm 2.7^{**}$	36.3 ± 2.4

**Significantly different from vehicle, $P < 0.01$. Values are given as the mean $\pm$ S.E.M. of five experiments.

Table 3. Effect of NS 1619 on cigarette smoke-induced and substance P-induced rat tracheal plasma extravasation

		Evans blue dye ($\mu\text{g/g}$ tissue)	
		Cigarette smoke	Substance P
Control		47.3 ± 3.1	44.7 ± 2.5
NS 1619	1.0 mg/kg	$14.7 \pm 2.6^{**}$	42.1 ± 3.1

**Significantly different from vehicle, $P < 0.01$. Values are given as the mean $\pm$ S.E.M. of five experiments.

Discussion

Marijuana produces a myriad of pharmacological effects in animals or humans; and its anxiolytic, sedative, analgesic, and psychedelic properties are mainly attributable to the interactions of the active components of the drug with specific cannabinoid receptors, at least two types of CB₁ and CB₂. The activation of CB₁ receptors located in the central nervous system could mediate the central effects of the psychoactive components of marijuana (20, 21). These findings support the view that cannabinoids play an important role in the modulation of cortical neurotransmission (22) and cause the inhibition of acetylcholine, glutamate, dopamine, noradrenaline, and GABA release from brain slices (10). On the other hand, in the peripheral mechanism, cannabinoids suppress nociception resulting from tissue injury and inflammation (23, 24). Cannabinoids also act locally to suppress capsaicin-evoked immunoreactive calcitonin gene-related peptide release in rat hind paw skin (23) and capsaicin-evoked hyperalgesia (25). Behavioral studies indicate that peripheral antihyperalgesic actions of cannabinoids are mediated by CB₁ receptors (23–25). However, CB₂ receptors are also implicated in the modulation of inflammation (26) and in the antinociceptive effects of cannabinoids (27). In the airway tissues, Patel et al. (28) reported that activation of the CB₂ receptors inhibited sensory nerve activation of guinea pig and human vagus nerve and the cough reflex in guinea pigs. In our previous study, we discovered that cannabinoid receptor agonists, WIN 55212-2 and JWH 133, reduced guinea pig bronchial contraction induced by the activation of C-fibers, which may be caused by the inhibition of voltage-dependent Ca²⁺ influx and release of substance P from sensory nerves by the activation of CB₂ receptors in vitro (18). In this study, WIN 55212-2 also inhibited airway inflammatory reactions, for example, capsaicin-induced guinea pig bronchoconstriction and cigarette smoke-induced rat tracheal plasma extravasation through the interaction with CB₂ receptors in vivo. Our present evidence well agreed with the reports described above about the peripheral CB₂ modulation of neurotransmission such as C-fibers.

The efferent function of C-fibers may be inhibited at a presumed presynaptic level via the activation of several receptor types. Agonists at μ -opioid, α_2 -adrenergic, GABA_B, histamine H₃, neuropeptide Y₂, and 5-HT_{1-like} receptors have all been shown to inhibit excitatory nonadrenergic noncholinergic bronchoconstriction in the guinea pig, implying an action on airway C-fibers. It has been proposed that the inhibition evoked by a number of these receptors (μ -opioid, α_2 -adrenergic,

neuropeptide Y₂, and 5-HT_{1-like}) is mediated via a common mechanism involving the opening of Maxi-K⁺ channels (29). Mackie and Hille (17) suggested that inhibitory effect of cannabinoids on N-type voltage-dependent Ca²⁺ channels might be mediated by its interaction with other ion channels coupled either directly or indirectly to G proteins, for example, potassium channels. Deadwyler et al. (30) have shown that WIN 55212-2 increases voltage-dependent potassium-A current in cultured hippocampal cells. In our previous study, we discovered Maxi-K⁺ channel blockers, iberiotoxin and charybdotoxin, reduced the inhibitory effect of cannabinoid agonists on electrical field stimulation-induced guinea pig bronchial smooth muscle contraction, but not the A-channel blocker dendrotoxin or the ATP-sensitive K⁺ channel blocker glibenclamide. We have indicated that WIN 55212-2 could induce the opening of Maxi-K⁺ channels via the interaction with CB₂ cannabinoid receptors in vitro. Thus, in this study, the Maxi-K⁺ channel opener NS 1619 significantly reduced capsaicin-induced guinea pig bronchoconstriction and cigarette smoke-induced rat tracheal plasma extravasation. According to these results, WIN 55212-2 might induce the activation of Maxi-K⁺ channels by the interaction with CB₂ cannabinoid receptors and inhibits the airway inflammatory reactions caused by the activation of C-fibers in vivo. We could conclude that CB₂ cannabinoid receptors might physiologically interact with Maxi-K⁺ channels existing on the presynaptic terminal of C-fibers and negatively modulated their activation in airway tissues through the same mechanism as other receptors, for example, μ -opioid, α_2 -adrenergic, neuropeptide Y₂, and 5-HT_{1-like} receptors.

When excitatory C-fibers are stimulated, not only substance P but other neuropeptides also (e.g., neurokinin A and calcitonin gene-related peptide) are released from their nerve endings and exert various respiratory reactions (31). The inflammatory effects of these neuropeptides on airway tissues may be of pathological relevance in human bronchial hyperreactivity and we showed that anti-asthmatic compounds, disodium cromoglycate and nedocromil sodium, inhibited hyper-tonic saline-induced plasma extravasation in guinea pig airways by the inactivation of C-fibers (7). We suggest that CB₂ receptors, which physiologically inhibit the activation of C-fibers and the release of neuropeptides from their endings via the opening of Maxi-K⁺ channels, might be involved in the clinical effects of anti-asthmatic compounds described above.

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